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Epstein-Barr Virus Expression Within Keratinizing Nasopharyngeal Carcinoma

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Three stages of maturation can be seen in keratinizing nasopharyngeal carcinomas. These stages are similar morphologically to basal cells, intermediate and superficial squamous cells seen in normal squamous epithelium. Taking advantage of such a diverse tumour cell population, 10 keratinizing nasopharyngeal carcinoma (NPC) were examined by in situ hybridization for the presence of latent Epstein-Barr Virus (EBV) using EBV encoded RNAs (EBERs) and by immunohistology for the presence of EBV early antigen-diffuse (EA-D) and the 350/220 kd membrane glycoprotein of the EBV. The basal cell-like tumour cells are mainly infected latently with the virus; viral replication was found in isolated intermediate squamous cells, whilst superficial squamous cells are largely depleted of all the viral markers. We used a control series of non-keratinizing nasopharyngeal carcinomas composed of undifferentiated and poorly differentiated tumour cells and EBV latency was present in these tumours. Viral replication was detected by RT-PCR, in the undifferentiated tumours but viral replication was not seen by immunohistology. The possible relationship between EBV life cycle in these tumours and tumour cell differentiation is discussed in the light of these findings. ***J. Med. Virol. 55:227–233, 1998.***

KEY WORDS: EBV expression; NPC differentiation

INTRODUCTION

Epstein-Barr virus (EBV) is a human herpesvirus which persists in the oral cavity as a chronic low grade infection [Gerber et al., 1972; Sixbey et al., 1984]. There is limited knowledge on the mechanisms of this viral persistence [Yao et al., 1989a,b]. It has been proposed that the pool of the virus is maintained in a latent state in the pluripotent basal cells and virus replication takes place co-ordinately with epithelial cell differentiation [Allday et al., 1988; Klein 1989]. Indeed, exfoliated squamous cells have been shown to support limited virus replication [Sixbey et al., 1984; Lung et al., 1985], and in an epithelial cell culture model developed recently, viral replication correlated with differentiation [Li et al., 1992; Pauline et al., 1996], and viral latency associated with an impaired ability of the host cells to undergo differentiation [Niedobitek et al., 1994]. In human tissues, latent infection is typically present in undifferentiated nasopharyngeal carcinoma (NPC), whilst lytic infection is found in hairy leukoplakia [Greenspan et al., 1985]. In hairy leukoplakia, active virus replication is found in cells which are at a more advanced state of differentiation [Greenspan et al., 1985; Niedobitek et al., 1991].

Although viral latency predominates in NPC [Raab-Traub et al., 1992; Young et al., 1988], a recent study by Martel-Renoir et al. [1995], suggested that a low level of viral replication does occur in these tumour tissues. In their study the tumours were mainly undifferentiated carcinomas. In the current study, we investigated a series of 10 keratinizing nasopharyngeal carcinomas to see if viral presence in these tumours was related to keratinization and therefore differentiation.

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MATERIALS AND METHODS

For the purpose of the present study, NPC was classified on the basis of keratinization [Shanmugaratnam et al., 1991]. Formalin-fixed keratinizing carcinomas were obtained from the archives of the Department of Pathology, Sun Yat-sen University of Medical Sciences, Guangzhou, P.R.C. The non-keratinizing tumours were fresh biopsies obtained from NPC patients before treatment at Queen Mary Hospital, Hong Kong. These tumours were kept in liquid nitrogen until use. The EBV positive lymphoblastoid cell line, B95-8 and Raji, and the EBV negative cell line, BJAB, were cultured in RPMI-1640 containing 10% fetal calf serum and antibiotics.

The murine monoclonal antibodies specific for EBNA1 (OT1); the 138kd EA-D (OT13B); and the 350/220kd EBV membrane glycoprotein (OT6), and the rabbit antiserum (OT27-3) specific for the p18 EBV matrix protein were obtained from Organon-Teknika, the Netherlands. Optimal dilution of these antibodies was determined against formaldehyde fixed sections of B95-8, Raji and BJAB. The EBNA1 specific antibody was used at a final dilution of 1:300, the EA-D specific antibody, at a final dilution of 1: 400; and the 350/220 kd glycoprotein specific antibody, at a final dilution of 1:100. The p18 matrix protein specific antiserum was used at a final dilution of 1:150. None of these antibodies and antisera was reactive against sections of BJAB. The MA 350/220 kd glycoprotein and EA-D specific antibodies were reactive against fixed sections of B95-8 exclusively and the EBNA1 specific antibody was reactive against fixed sections of B95-8 and Raji cells.

Immunohistology was performed using the LSAB detection kit (DAKO, Denmark). Antigen retrieval was optimized by microwaving sections in distilled water for 10 min. The sections were then washed with 1 × PBS and incubated in blocking solution (containing carrier protein and 15 mM sodium Azide). The sections were allowed to react overnight at 4°C with antibodies at the indicated dilution, washed, and then allowed to react for 30 min at 37°C with biotinylated anti-rabbit or anti-mouse immunoglobulin followed by horse raddish lactose peroxidase-conjugated streptavidin and substrate solution according to the manufacturer's instructions. Sections were counter-stained with methyl green. As the biopsy material contained both tumour and non-tumour an inbuilt negative control was present, but duplicate sections of tumour were also stained omitting the primary antibody.

For detection of EBERs, tissue sections were deparaffinized as before and treated with 100 μg per ml of proteinase K at 37°C for 30 min. After washing with DEPC treated water, tissue sections were incubated at 37°C for 2 hours with an FITC conjugated probe (Dako), followed by alkaline phosphatase conjugated anti-FITC and a substrate solution (Dako) for 30 min at room temperature, according to the manufacturer's instructions. All the reactions were carried out in the dark and tissue sections were counter-stained with 1% methyl green.

Fresh NPC biopsies were obtained from Department of Radiotherapy, Queen Mary Hospital. For RT-PCR RNA was extracted and selected by Micro Prep mRNA purification kit (Pharmacia, Gaithersburg, MD) according to the manufacturer's instructions. Single stranded cDNAs were obtained by reverse-transcribing the oligo-dT selected mRNA in 1 × RT buffer containing 0.4 mM dNTPs, 0.5 μg random primers, 10 units RNasin, and 15 units reverse transcriptase (MAMV) at 42°C for 1 hour. One μl of the first-strand cDNA was used for RT-PCR. The primers used are as described in Table I and PCR amplification was carried in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 2 mM MgCl2) containing 0.25 mM of each of the dNTP and 1.2 units of Taq DNA polymerase. The reaction was carried out for 30 cycles at 94°C for 1.5 minutes, 57°C for 1 minute, and 72°C for 2 minutes. For nested PCR, 1 μl of first of round reaction product was amplified under same condition. PCR products were electrophoresed on 2% agarose and visualized by ethidium bromide staining.

RESULTS

Keratinizing NPC comprises a mixture of tumour cells (Fig. 1a) with cells present resembling undifferentiated or poorly differentiated basal cells (B), keratinizing intermediate squamous cells (I), and superficial squamous cells (S). The basal tumour cells type have a high nucleus to cytoplasm ratio and poorly defined cell borders. The intermediate squamous cells have an decreased nucleus to cytoplasm ratio and often show intercellular bridges with well defined borders. The superficial squamous type cells flattened with eosinophilic cytoplasm and small nuclei. The superficial and intermediate cells aggregate giving the appearance of 'pearls' which is a characteristic feature of keratinizing NPC.

To relate EBV activity with tumour differentiation, we determined the distribution of viral markers representative of viral latency and replication, respectively, between these three types of tumour cells. Typically, EBERs were found regularly in basal tumour cells and some intermediate squamous type (Fig. 1b). A significant population of intermediate squamous tumour cells and most of the superficial tumour cells were not positive for EBERs. EA-D was located to the nuclei of few isolated foci, and these positive cells showed prominent nucleoli (Fig. 1c). The decreased nucleus to cytoplasm ratio of these reactive cells and the smaller sizes of their nuclei are features of intermediate squamous cells. The 350/220 kd membrane glycoprotein was detected in isolated foci (Fig. 1d), and this reaction was typically located to the membrane of the intermediate squamous tumour cells.

Table II summarizes the distribution of EBERs, EA-D, and the EB membrane glycoprotein in 10 keratinizing NPCs. EBERs were present regularly in basal cells

TABLE I. Sequences and Locations of Primers for RT-PCR*

Primer	Sequences	Coordinates(3′)	Sizes of cDNA	Sizes of DNA
Z1	5′-AGCAGACATTGGTGTGCACAG-3′	102713	252	450
Z2	5′-ACATCTGCTTCAACAGGAGG-3′	102304		
Z3(nested)	5′-ACGACGCACACGGAAACCACAAC-3′	102667	187	386
Z4(nested)	5′-GCGCAGCCTGTCATTTTCAG-3′	102323		
L1	5′-GTGGATGTGGAACTGTTTCAG-3′	89957		
L2	5′-CTGTATCCAG\CCGCGGATGTCAC-3′	90734		
L3(nested)	5′-AACTGTTTCCAGGGCCTGAC-3′	89965	204(gp220)	795
L4(nested)	5′-ATGTCACCAGCCCAACACCAG-3′	90720		
R1	5′-GATGTTGAGCGTGGCCATTAG-3′	104960		
R2	5′-CCATACAGGACACAACACCTC-3′	106146		
R3(nested)	5′-CATTAGCCCGCCCCATTCTC-3′	104994	238	1181
R4(nested)	5′-CAACACCTCACTACACAAAC-3′	106135		
M1	5′-AGAGACTCTCCCGAACTAGCAG-3′	84255	240	346
M2	5′-ATGCCGAGGTAGGGGTTATGAC-3′	83952		
M3(nested)	5′-CTAGCAGCATTTCCTCCAAC-3′	84242	168	275
M4(nested)	5′-GGAGTTGGATCTTCATCCTCC-3′	84007		

*The sizes of cDNA and genomic DNA were based on the sequence of B95-8 strain [Baer et al., 1984; P Farrell, 1990], and the number represents position of 3′ end of nucleotide as refers to the sequence on B95-8.

of all the tumours and it was variously present in intermediate squamous tumour cells, but it was not detected in superficial tumour cells. EA-D was detected in small isolated foci of intermediate squamous cells or cells which resemble both basal and intermediate tumour cells in seven tumours. EB membrane glycoprotein was detected in isolated small foci in eight tumours. The positive cells were mainly intermediate squamous type and occasionally cells that also resemble basal cells. The sections were also tested for EBNA1 and 18 kd matrix protein, the latter being a component of viral capsid protein (VCA) [van-Grunsven et al., 1994]. The distribution of EBNA1 (Fig. 1e) was similar to EBERs and that of EB matrix protein, to the membrane glycoprotein.

Non-keratinizing carcinomas reacted regularly for EBERs and EBNA1, and except for an occasional isolated cell, these tumours did not show foci of reactivity for EA-D, MA, or VCA when tested by immunohistochemistry under similar conditions (results not shown). Martel-Renoir et al. [1995] showed recently that non-keratinizing tumours exhibit low level of lytic EBV transcription. In agreement with these researchers, 10 of the 11 non-keratinizing tumours we tested showed transcription from exon 1 of BZLF1 after the first round of amplification and in the remaining tumour, after a second round of amplification (Fig. 2a). The resulting PCR products were distinguished from the larger genomic DNA (Fig. 2). Transcription from 2 early lytic cycle genes, i.e., BRLF1 and BMLF1, are shown in Figures 2b and c, respectively. The BRLF1 transcript was detected in nie tumour biopsies only after a second round of amplification (Fig. 2b). The PCR product corresponds to Rts, but, the transcript previously reported to be present in some B cell lines due to alternate splicing (RAZ), was not detected in these tissues. The BMLF1 transcript was detected in three biopsies after the first round amplification and in five other biopsies after a further amplification. The PCR product of the transcript of BLLF1 encoding the 220 kd truncated form of membrane glycoprotein was detected in nine tumour biopsies (Fig 2d). All the above described EBV transcripts were detected in a NPC xenograft (NM1530) and B95-8 cell line as the first round PCR products. The BARF transcript [Chen et al., 1992] was present in abundance in all the specimens tested (not shown). The pattern of transcription in these tumours (Table III) confirm the earlier findings of Martel-Renoir et al. [1995] showing that productive EBV replication occurs commonly, albeit at a low level, in NPC tumours.

DISCUSSION

The World Health Organization (WHO) in its latest classification of NPC has two categories of tumours; keratinizing and non-keratinizing carcinomas. The keratinizing carcinomas or SCC are uncommon [Zong et al., 1983]. Within this tumour the cells range from large and basophilic to small and eosinophilic. Past studies of SCCs have shown the tumour proliferative activity, as determined by PCNA intensity, is most in the basal type cells and least in the superficial cells [Lorz et al., 1994; Jones et al., 1994].

In this study, advantage was taken of the heterogeneous tumour cell population of keratinizing NPC to relate EBV latency and replication to tumour growth and differentiation. Because keratinizing tumours are uncommon and rarely diagnosed on clinical criteria alone, we used fixed tumour tissues from our archives. Viral latency was indicated primarily by the presence of EBERs and further evaluated using an EBNA1 antibody. Early and late virus replication was studied primarily using an EA-D antibody and an antibody specific for the 350/220 kd membrane glycoprotein, and the results were further assessed using an antibody specific for the 18kd EBV matrix protein [van-Grunsven et al., 1994]. Selection of these antibodies was based on immunoblotting results and after they had been evaluated against fixed LCLs. The antibodies

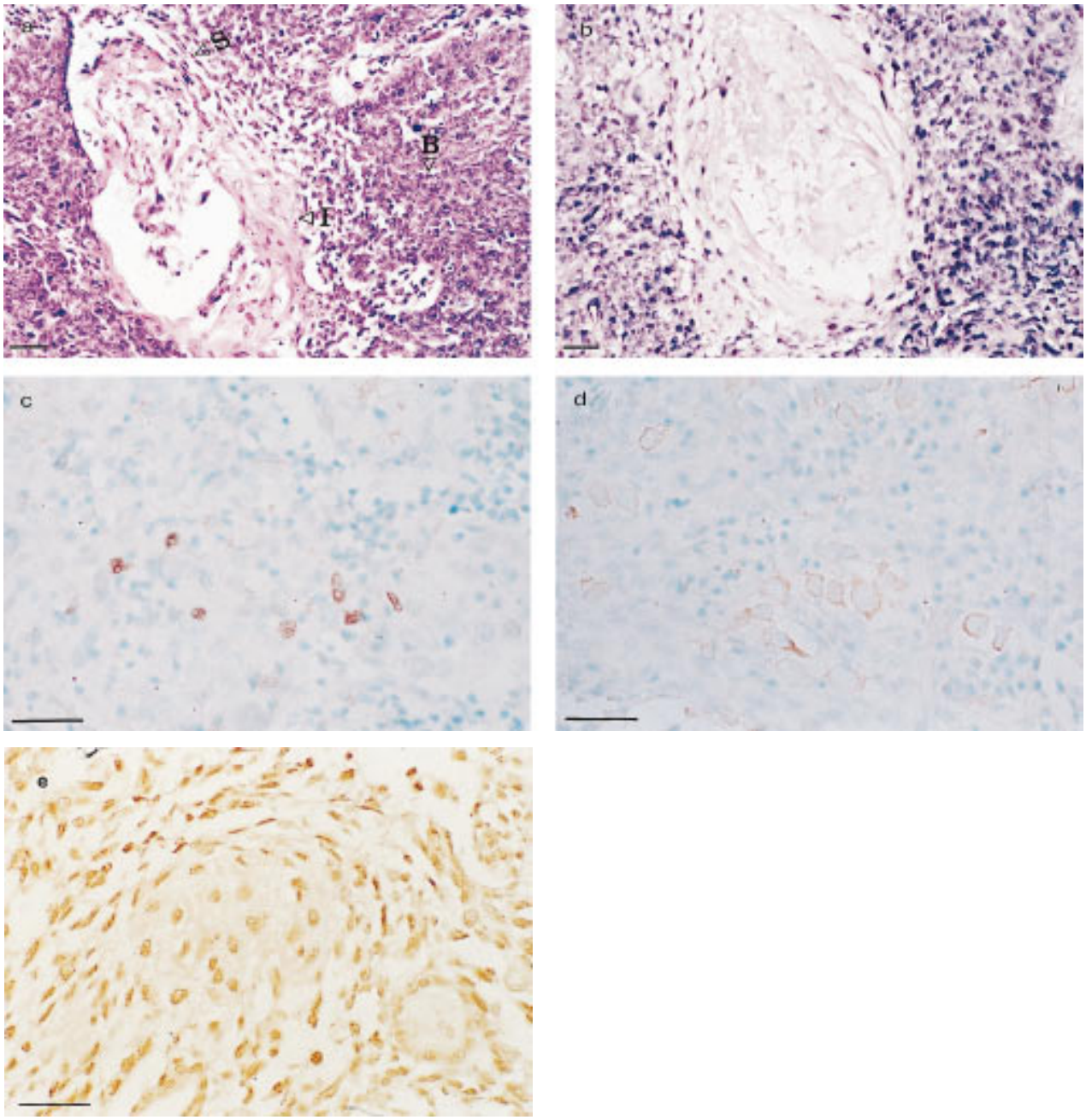

Fig. 1. Localization of EBV latency and EBV replication in keratinizing NPC. Paraffin embedded sections of keratinizing tumours were examined by H & E staining **(a),** in situ hybridization for the presence of EBERs **(b),** or were allowed to react with antibodies specific for EA-D **(c),** the 350/220 kd glycoprotein **(d)** and EBNA1 **(e).** Note the heterogeneous tumour cell population in the H & E stained section in (a) that the mixture variously comprise of tumour cells with similar morphological features as basal cells (B), intermediate squamous cells (I), and superficial squamous cells (S); and that EBERs were predominantly localized to and regularly present in the basal cells, some intermediate squamous cells, or cells which resemble both intermediate and basal cell; EA and MA occurred in small clusters of intermediate squamous cells; and the absence of all of these viral markers in the superficial squamous cells. Scale bar = 50 μ.

specific for EA-D and MA were similarly reactive against fixed LCL as they were against fresh LCLs.

Non-keratinizing carcinoma, particularly undifferentiated NPC has been associated consistently with EBV with the localisation of viral genomes to the malignant epithelial cells [Klein 1979; Pagano 1992]. However, the evidence regarding the possibility of an EBV association with keratinizing squamous cell NPC has been conflicting. The studies from Raab-Traub et al. [1987] and other laboratories [Pathmanathan et al., 1995; Chen et al., 1993; Dickens et al., 1992; Akao et al., 1991] indicated that keratinizing NPC from Asian patients are associated regularly with EBV. But Niedobitek et al. [1991] failed to detect the virus in such

TABLE II. Distribution of EBERs, EBNA1, EA-D, MA, and VCA in Keratinizing Nasopharyngeal Carcinoma*

	EBERs			EBNA1			EA-D			MA (gp 350/220)			VCA		
Tumours	B	I	S	B	I	S	B	I	S	B	I	S	B	I	S
68642	+			+	(+)			(+)			(+)			(+)	
90661	+			+				(+)			(+)			(+)	
16064	+			+							(+)			(+)	
112571	+	(+)		+	(+)			nd			(+)			(+)	
Y034	+	(+)		+	(+)			(+)			(+)			(+)	
105312	+	(+)		+	(+)			(+)			(+)			(+)	
42437	+	(+)		+							(+)			(+)	
84757	+			+				(+)							
205091	+			+	(+)			(+)			nd			(+)	
191237	+	(+)		+				(+)			(+)			nd	
Total	10	5	0	10	5	0	0	7	0	0	8	0	0	8	0

*Three stages of maturation were identified; those resembling basal cells (B), intermediate squamous cells (I), and superficial squamous cells (S) in the normal epithelium. Markers detected in isolated foci indicated by parenthesis.

TABLE III. Detection of EBV Replicative Genes Transcriptions by RT-PCR*

NPC	BZLF 1		BRLF 1		BMLF 1		BHRF 1		BLLF 1		BamHI A
Biopsies	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st
NPC114	+		−	+	+	+	−	+	−	+	+
NPC115	+		−	+	−	+	−	+	−	+	+
NPC116	+		−	+	+	+	−	+	−	+	+
NPC133	+		−	+	−	+	−	−	−	−	+
NPC137	+		−	−	−	−	−	−	−	−	+
NPC142	+		−	+	−	+	−	+	−	+	+
NPC150	+		−	+	−	+	−	+	−	+	+
NPC153	+		−	−	−	−	−	−	−	+	+
NPC154	−	+	−	+	−	−	−	+	−	+	+
NPC159	+		−	+	+		−	+	−	+	+
NPC161	+		−	+	−	+	−	+	−	+	+
NM1530	+		+		+		+		+		+
B95-8	+		+		+		+		+		+

*EBV transcripts detected (+) by RT-PCR after 1st or 2nd round of amplification as described in text. BZLF1 and BRLF1: immediately early lytic genes encode ZEBRA and Rta. BMLF1 and BHRF1: early lytic genes encode. BLLF1: late lytic gene encode gp350/220. BamHI A mRNA: abundant EBV transcripts in NPC cells. NM 1530 is an nude mouse NPC tumour.

tumours from Caucasian patients, and similar observation were also obtained from some other groups [Hording et al., 1993; Nicholls et al., 1997; Klein et al., 1974]. In the present study the findings that keratinizing NPC from Chinese patients are EBV related were confirmed and further it was shown that these tumours exhibited a range of EBV expression. Viral latency was found to be the dominant virus-cell interaction in basal tumour cells. These cells were positive regularly and intensely for EBERs and most of them were also positive for EBNA1. The differentiating intermediate squamous tumour cells were stained less intensely for EBERs and EBNA1 and a proportion of these tumour cells were negative for EBV. The superficial cells were uniformly negative for EBERs and EBNA1. Such patterns of distribution of these viral markers suggests that the EBV genome can persist in basal and intermediate cells but eventually lost as tumour cells differentiate. However, this disruption of viral latency did not appear to lead commonly to activation of the replication cycle, because only small isolated foci of cells were reactive for EA-D and membrane glycoprotein. This site of viral replication appeared mainly in intermediate squamous cells. Though viral replication has been seen in undifferentiated carcinomas, this is the first report of replication in keratinizing carcinomas. The distribution of the latent and lytic viral markers in these tumours suggests that the EBV life cycle is related to tumour cell differentiation.

The findings of rare lytic expression in keratinizing cells is important in considering the life cycle of EBV in healthy populations. The normal oropharyngeal mucosa of carriers has been reported to be infected chronically with EBV [Sixbey et al., 1984], and virus replication has been observed in stratified squamous epithelium [Allday et al., 1988; Klein, 1989; Miller, 1990], but it is not known how this infection can be been sustained in such constantly self-renewing tissues as no studies to date have shown conclusively that basal cells of normal oropharyngeal epithelium consistently show the virus. It has been postulated that latently infected B cells may be the reservoir of EBV [Datta et al., 1980; Gratama et al., 1990; Yao et al., 1989a,b; Tao et al., 1995], but our previous report demonstrated that the populations of EBV in blood and mucosa are genetically and phenotypically distinct [Chen et al., 1996]. Fur-

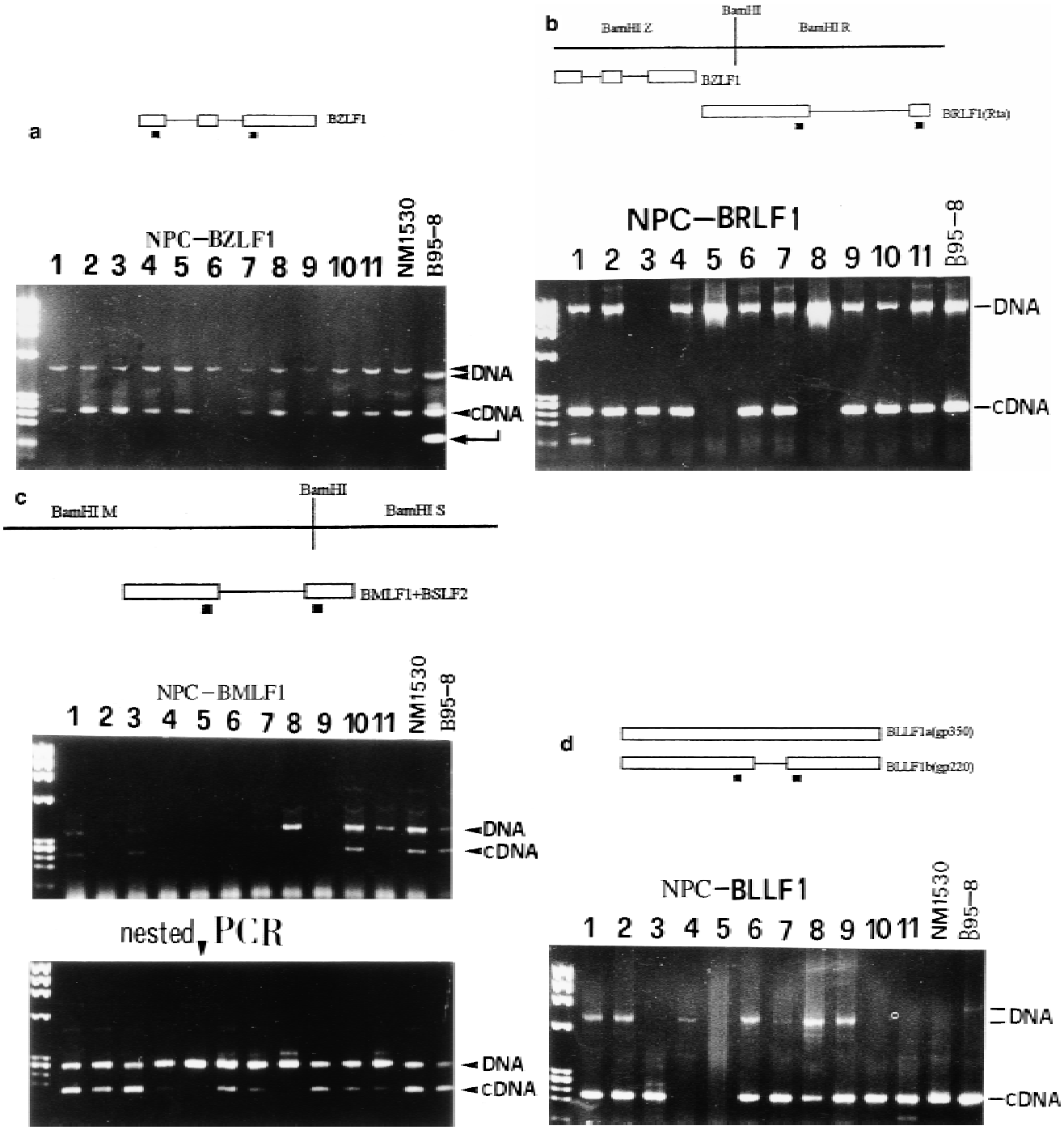

Fig. 2. EBV replication in non-keratinizing NPC. Transcripts of BZLF1 **(a),** BRLF1 **(b),** BMLF1 **(c)** and the spliced transcript of BLLF1 **(d)** were detected in 11 NPC biopsies, an NPC xenograft (NM1530) and B95-8 cell line as described in Materials and Methods.

thermore, healthy carriers harbour the same genotypes for protracted period [Lung et al., 1996], although virus harboured by different individuals are genetically distinct. This would argue against the possibility that repeated infection with exogenous virus may serve to perpetuate the chronic infection in the epithelium. Therefore, the role of epithelial cells in the life-long persistence of EBV cannot be excluded. Further studies are required to clarify the status of EBV lytic infection in normal epithelium.

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